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Evaluation of antiviral activities of *Houttuynia cordata Thunb*. extract, quercetin, quercetrin and cinanserin on murine coronavirus and dengue virus infection

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ABSTRACT

Objective: To evaluate the *in vitro* activities of the ethyl acetate (EA) fraction of *Houttuynia cordata* (*H. cordata*) Thunb. (*Saururaceae*) and three of its constituent flavonoids (quercetin, quercitrin and rutin) against murine coronavirus and dengue virus (DENV).
Methods: The antiviral activities of various concentrations of the EA fraction of *H. cordata* and flavonoids were assessed using virus neutralization tests against mouse hepatitis virus (MHV) and DENV type 2 (DENV-2). Cinanserin hydrochloride was also tested against MHV. The EA fraction of *H. cordata* was tested for acute oral toxicity in C57BL/6 mice.
Results: The EA fraction of *H. cordata* inhibited viral infectivity up to 6 d. Cinanserin hydrochloride was able to inhibit MHV for only 2 d. The 50% inhibitory concentrations (IC_{50}) of the EA fraction of *H. cordata* added before the viral adsorption stage were 0.98 μg/mL for MHV and 7.50 μg/mL for DENV-2 with absence of cytotoxicity. The mice fed with the EA fraction up to 2000 mg/kg did not induce any signs of acute toxicity, with normal histological features of major organs. Certain flavonoids exhibited comparatively weaker antiviral activity, notably quercetin which could inhibit both MHV and DENV-2. This was followed by quercitrin which could inhibit DENV-2 but not MHV, whereas rutin did not exert any inhibitory effect on either virus. When quercetin was combined with quercitrin, enhancement of anti-DENV-2 activity and reduced cytotoxicity were observed. However, the synergistic efficacy of the flavonoid combination was still less than that of the EA fraction.
Conclusions: The compounds in *H. cordata* contribute to the superior antiviral efficacy of the EA fraction which lacked cytotoxicity *in vitro* and acute toxicity *in vivo*. *H. cordata* has much potential for the development of antiviral agents against coronavirus and dengue infections.

1. Introduction

Severe acute distress syndrome (SARS) is a highly contagious respiratory illness caused by SARS coronavirus, which emerged in 2003 and rapidly spread throughout the world, with a mortality rate of 10%–15%. Although the disease disappeared in mid-2003, its re-emergence cannot be excluded since SARS-like coronaviruses are zoonotic and exist in animal reservoirs (*e.g.*, bats, raccoon dogs and palm civets), thus posing a potential risk for future epidemics [1–3]. SARS coronavirus is a large, enveloped, single-strand, positive-sense RNA virus. The viral genome is about 30 kbp, containing open reading frames that encode the polymerases required for viral RNA synthesis, while the remaining open reading frames encode structural proteins

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(*i.e.*, spike, envelope, membrane and nucleocapsid) and accessory proteins.

First identified in 2012, the emerging Middle East respiratory syndrome (MERS) coronavirus causes severe pneumonia with multiorgan involvement (including acute renal failure), and a mortality rate of 36% (446 deaths out of 1 244 cases documented by WHO as of 9 June 2015). It is considered to be a zoonotic virus being transmitted from camels in the Middle East. Currently, no specific treatment exists against MERS coronavirus.

The murine coronavirus, mouse hepatitis virus (MHV), is a coronavirus that causes an epidemic murine illness with high mortality. Generally, MHV is extremely infectious to colonies of mice and causes hepatitis upon infection. SARS, MERS and MHV belong to the group 2 coronaviruses and are classified under the genus *Betacoronavirus*. In view of the relatedness of these coronaviruses, MHV was selected in this study to act as a surrogate model for SARS and MERS coronaviruses which necessitate BSL-3 facilities, whereas MHV is considered a BSL-2 pathogen.

Belonging to the genus *Flavivirus*, dengue virus (DENV) represents the most important mosquito-borne viral disease with considerable resurgence in many parts of the world. There are four antigenically-related but distinct DENV serotypes, such that infection with one serotype does not confer life-long immunity against the other serotypes. DENV infection causes dengue fever, and occasionally the more serious conditions of dengue hemorrhagic fever and dengue shock syndrome [4]. Antibody-dependent enhancement is thought to play a central role in dengue pathogenesis, with the risk of developing dengue hemorrhagic fever and/or dengue shock syndrome being greater in secondary infections with DENV-2 compared to other serotypes [5]. The different manifestations of dengue may also be attributed to DENV variants with varying degrees of virulence [6], while viral load is also a contributing factor in the development of potentially fatal complications [7]. In addition, being a highly prevalent serotype in tropical and subtropical regions worldwide, DENV-2 was selected for this study. Although several dengue candidate vaccines are undergoing clinical trials, there are currently no effective antiviral therapies which are urgently needed to control dengue.

Belonging to the *Saururaceae* family, *Houttuynia cordata* (*H. cordata*) Thunb. is a perennial plant native to mountainous regions of eastern Asia, with an indefinite spread as a creeping rhizome in moist locations. This herb possesses very promising antiviral properties especially against clinically important enveloped viruses such as herpes simplex virus-1 (HSV-1), influenza virus, and human immunodeficiency virus-1 *in vitro*. Interestingly, the steam distillate of *H. cordata* can strikingly inactivate an enveloped virus but is incapable of inactivating a non-enveloped virus [8–12].

To support these observations, we and others have demonstrated that the ethyl acetate (EA) fraction of *H. cordata* is effective in inhibiting the infectivity of enveloped viruses such as DENV [13]. Meng *et al.* discovered 11 common peaks in the HPLC-DAD MS fingerprint of fresh *H. cordata* [14]. In our previous project, we verified some of these peaks in the EA extract as polyphenols or flavonoids (chlorogenic acid, hyperoside, quercetin and quercetrin) and have investigated their antiviral efficacy against DENV [13]. Flavonoids are a class of natural products with high pharmacological potency, are ubiquitous in photosynthesizing cells and hence likely to be consumed daily [15]. Flavonoids are known to display antiviral activities, *e.g.*, glabranine and 7-O-methyl-glabranine against dengue virus, procyanidin and pelargonidin against HSV, and catechins against influenza virus [16,17].

Using virus-specific neutralization tests, this study tested the EA fraction of *H. cordata* for its antiviral efficacy against MHV (for the first time) and DENV-2 (as further verification using a different batch of *H. cordata* specimen). Three flavonoid components of *H. cordata*, *i.e.*, quercetin, quercitrin and rutin, were also investigated for their antiviral activities against both viruses. They were also selected since they are common and naturally occurring flavonoids, whose molecular structures share high degrees of similarity. In the MHV experiments, we also compared the potency of cinanserin hydrochloride which has been proven to neutralize SARS coronavirus *in vitro*. All the compounds were also tested for cytotoxicity *in vitro*, while the EA fraction was also tested for acute oral toxicity in mice.

2. Methods and materials

2.1. Plant material, ethanolic extraction, aqueous-EA fractionation, and flavonoids of H. cordata

The aerial parts (fresh leaves) of *H. cordata* were collected from a farm in Johor, Malaysia and authenticated in the Department of Pharmacology, National University of Singapore. The voucher specimen of *H. cordata* was deposited in the Singapore Botanic Gardens and assigned the identification number SING2011-203. Prior to extraction, the herb (dry weight of 500 g) was washed with de-ionized water, homogenized to a fine powder, and soaked overnight in 80% (v/v) ethanol. The next day, the ethanolic extract was removed and stored. More solvent was added to the blended herb, which was left to soak till exhaustion. The extract was filtered, concentrated with a rotary evaporator (Buchi Rotavapor R-144) and freeze-dried to yield 19.28 g of crude extract in powder form. The crude extract was then dissolved in EA and de-ionized water. The water and EA phases were separated with a separating funnel. The EA phase was concentrated with a rotary evaporator, and the concentrated EA fraction was subsequently stored at −80 °C overnight and freeze-dried. The flavonoids tested were quercetin dihydrate (≥98% HPLC), quercitrin, and rutin hydrate (95%), all purchased from Sigma–Aldrich.

2.2. Mouse hepatitis virus, cell culture, and neutralization test

CCL9.1, a normal *Mus musculus* (mouse) liver epithelial cell line, was used for propagation of MHV and for the MHV neutralization test. Compounds that were tested against MHV were the EA fraction of *H. cordata*, its flavonoid components, and cinanserin hydrochloride (Tocris Bioscience). Each compound was 2-fold serially diluted with the corresponding diluent, and the dilutions were then added to wells of a 96-well microtiter plate. Next, 100 $TCID_{50}$ of MHV was added to each diluted compound and incubated at 37 °C for 1 h with 5% CO_2. Also tested was treatment with diluent only at various concentrations. Virus-infected controls and uninfected cell controls were included in each batch of assays. Confluent CCL9.1 cells were cultured in DMEM supplemented with 10% horse serum, and 1×10^5 cells were seeded into each well of another 96-well

microtiter plate, and incubated at 37 °C with 5% CO_2 until the cells were 80% confluent. Cell culture fluid from each well was discarded. Each virus-compound mixture and controls were transferred to these wells in duplicate, followed by addition of DMEM with 10% (v/v) horse serum. The plate was sealed and incubated at 37 °C with 5% CO_2, and observed daily for 6 d. The highest dilution of compound that inhibited cytopathic effects (CPE) was considered as the minimum inhibitory concentration (MIC).

2.3. Dengue virus, cell culture, and plaque reduction neutralization test

The New Guinea C strain of DENV-2 was propagated in the C6/36 *Aedes albopictus* mosquito cell line and maintained in Leibovitz L15 medium supplemented with 3% (v/v) fetal bovine serum at 28 °C under 5% CO_2 atmosphere. The anti-DENV-2 activity of *H. cordata* and its flavonoids were evaluated by plaque reduction neutralization test or PRNT [18,19]. BHK-21 (baby hamster kidney) fibroblasts were cultured to form cell monolayers in 24-well plates with RPMI-1640 supplemented with 10% (v/v) fetal bovine serum at 37 °C under 5% CO_2. The test compounds were dissolved in the relevant diluents, and 2-fold serial dilutions were prepared to obtain different concentrations. DENV-2 New Guinea C neutralizing monoclonal antibody 3H5 from Chemicon served as the positive control [20]. Diluent only (at various concentrations), virus, and cell controls were also included by adding the corresponding diluent, virus suspension, and medium without the treatment compounds. Each experiment was performed in duplicate. DENV-2 (90 plaque-forming units or PFU) were incubated for 1 h with various concentrations of each compound together with controls before adding to the cells. The virus-sample mixtures were incubated with the cells at 37 °C under 5% CO_2 for another hour with rocking at 15-min intervals before the cells were overlaid with 1% carboxymethylcellulose at 37 °C under 5% CO_2 for 6 d. The cells were then fixed with 20% formaldehyde and stained with 1% crystal violet, and the number of plaques was counted. The percentage plaque reduction of the compounds at every dilution was determined as follows: (mean number of plaques in virus control) − (average number of plaques in sample) × 100% divided by (mean number of plaques in virus control). The percentage plaque reduction was plotted against various concentrations of the test agents to determine the concentration that causes 50% plaque reduction (IC_{50}).

2.4. MTT cell viability and proliferation assay

The MTT cell proliferation assay was performed to determine the cell viability following exposure to the test compounds. Various concentrations of each test compound were added to wells containing cell monolayers and incubated at 37 °C under 5% CO_2 for 48 h. After incubation, MTT reagent was added to each well, and further incubated for 4 h or until purple precipitates were visible under an inverted microscope. Then, 100 μL of 100 g/L SDS in 0.01 mol/L HCl were added to each well and incubated overnight in the dark at room temperature. The optical density (OD) at 570 nm was then read, and the cell inhibition rate calculated from the formula: [1 − (OD of sample with cells) − (OD of sample without cells) divided by (OD of solvent with cells) − (OD of medium without cells)] × 100%.

The inhibition rates were plotted against various sample concentrations to ascertain the concentration that causes 50% cytotoxicity (CC_{50}). The EA fraction and flavonoids (either individually or in combination) were tested at the same concentrations as those for neutralization tests. The assay included wells containing medium only as well as untreated control cells. Each experiment was repeated, and the mean and standard deviation were calculated.

2.5. Acute oral toxicity testing

This approach was adapted from the OECD Guideline for Testing of Chemicals, 420: Acute Oral Toxicity – Fixed Dose Procedure, and relied on the observation of clear signs of toxicity or even mortality. C57/BL6 nulliparous and non-pregnant female mice about 8-weeks old were obtained from the Laboratory Animals Center, National University of Singapore. Upon arrival, they were kept in cages for 5 d prior to commencement of the study. On the actual dose-feeding day, the mice were fasted for about 4 h. Doses of 100, 500, 1 000 and 2 000 mg/kg were prepared for administration using water as vehicle to suspend the ethanolic extract of *H. cordata* at a constant volume of 1 mL/100 g of body weight. In total, the five groups of mice (*n* = 2 each) were fed with a single dose of the extract (including the vehicle control) by oral gavage. A sighting study was then conducted frequently from the first 30 min, with special attention to the first 4 h. Thereafter, the condition of each mouse was observed daily to detect abnormalities such as changes in physical appearance, behavioral signs and body weight. All mice were finally euthanized after 14 d of observation, and major organs (brain, heart, lungs, liver and kidneys) were harvested for histopathological examination after staining with hematoxylin and eosin (H&E).

3. Results

3.1. Anti-MHV activities of EA fraction of H. cordata, quercetin and cinanserin

The EA fraction of *H. cordata* was evaluated at concentrations of 3.91 μg/mL down to 0.24 μg/mL. Table 1 and Figure 1 show that the EA fraction of *H. cordata* exhibited anti-MHV activity at a MIC of 0.98 μg/mL without any apparent cytotoxic effects on CCL9.1 cells.

Tested at concentrations from 500.00 to 15.63 μg/mL, the anti-MHV effect of quercetin was significantly less, with MIC

Table 1

IC_{50}, CC_{50} and selectivity indices of *H. cordata* fraction, flavonoids, and cinanserin against MHV.

Sample	IC_{50} (μg/mL)	CC_{50} (μg/mL)	Selectivity index
EA fraction	0.98	NC[a]	>4.00[b]
Quercetin	125.00	116.52	0.93
Quercitrin	NIL	NC[a]	NIL
Rutin	NIL	NC[a]	NIL
Cinanserin (1 dpi)	31.25	NC[a]	>4.00[c]
Cinanserin (2 dpi)	62.50	NC[a]	>2.00[c]

[a] NC: No cytotoxicity at all concentrations tested against CCL9.1 cells.
[b] Highest selectivity index of EA fraction (*i.e.*, lowest concentration for viral inhibition coupled with absence of cytotoxicity). [c] Selectivity index with absence of cytotoxicity.
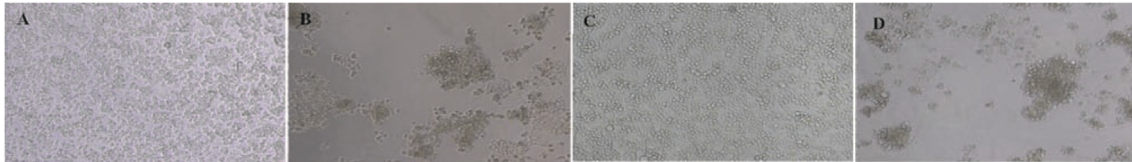
Figure 1. Neutralization of MHV infectivity by the EA fraction of *H. cordata* on 2 dpi.
(A) Uninfected control of CCL9.1 cells. (B) MHV-infected CCL9.1 cells control. (C) MHV-infected CCL9.1 cells subjected to 0.98 μg/mL of EA fraction. (D) MHV-infected CCL9.1 cells subjected to 0.49 μg/mL of EA fraction. Absence of obvious CPE in (C) indicates neutralization of MHV infectivity.

of 125.00 μg/mL (Figure 2), cytotoxicity CC_{50} value of 116.50 μg/mL, and selectivity index of 0.93. However, quercitrin and rutin did not exhibit antiviral activity on 2 days post-infection (dpi) at concentrations of 500.00 μg/mL and below (Table 1).

Cinanserin hydrochloride was tested at concentrations ranging from 125.00 to 3.91 μg/mL. Table 1 indicates that cinanserin exerted anti-MHV activity at MIC of 31.25 μg/mL on 1 dpi and 62.50 μg/mL on 2 dpi (Figure 3), with minimal or no cytotoxicity. However, on 3 dpi, no viral inhibition was observed.

3.2. Anti-DENV-2 activities of EA fraction of H. cordata, quercetin and/or quercetrin

The EA fraction of *H. cordata* was tested starting with the highest concentration of 166.67 μg/mL followed by 2-fold dilutions down to 0.08 μg/mL. By means of PRNT, a distinct trend of DENV-2 inhibition was observed at various concentrations of the EA fraction without cytotoxicity to BHK cells, with the IC_{50} being 7.50 μg/mL. The efficacy of the EA fraction in inhibiting DENV-2 infection was clearly evident at concentrations of 20.83 μg/mL and above, at which complete viral inhibition was achieved (Figure 4).

Individual flavonoids were tested at concentrations of 500.00 μg/mL down to 15.63 μg/mL. Table 2 indicates that quercetin displayed anti-DENV-2 activity with an IC_{50} of 176.76 μg/mL, CC_{50} value of 155.38 μg/mL, and selectivity index of 0.88. Although quercitrin showed less activity against DENV-2 with an IC_{50} of 467.27 μg/mL, this flavonoid did not cause cytotoxicity to BHK cells. Similar to experiments with

MHV, no anti-DENV-2 activity was detected with rutin treatment (without cytotoxicity).

Given that quercetin and quercitrin individually exhibited anti-DENV-2 properties, we evaluated whether the combination of both flavonoid compounds (at 1:1 ratio) could exert enhanced DENV-2 inhibition. Indeed, this combination of components achieved a synergistic anti-DENV-2 effect and lower cytotoxicity, with improved corresponding values of IC_{50} (158.21 μg/mL), CC_{50} (270.00 μg/mL), and selectivity index (1.71), as shown in Table 2.

3.3. Absence of non-specific virus inhibition and cytotoxicity of DMSO and aqueous alkali solvents

The EA fraction, quercetrin and rutin were dissolved in 1% DMSO. Quercetin was dissolved in aqueous alkali (0.06 mol/L NaOH) as it was not very soluble in DMSO. Various concentrations of EA fraction and compounds were subjected to the corresponding viral neutralization tests as well as the MTT assay. As negative controls, DMSO and aqueous alkali were also tested separately by DENV-2 PRNT, MHV neutralization test and the MTT assay which revealed absence of non-specific DENV-2 and MHV inhibition as well as lack of cytotoxicity of these solvents (data not shown).

3.4. Absence of acute toxicity and pathology in mice after oral feeding with H. cordata extract

Throughout the 14-day period of observation, the control group as well as the four groups of mice fed with varying doses
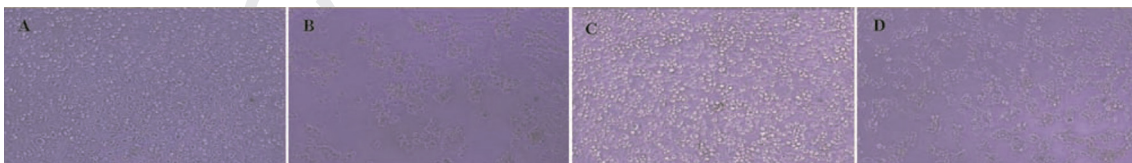

Figure 2. Neutralization of MHV infectivity by quercetin (dissolved in aqueous alkali) on 2 dpi.
(A) Uninfected control of CCL9.1 cells. (B) MHV-infected CCL9.1 cells control. (C) MHV-infected CCL9.1 cells subjected to 125 μg/mL of quercetin. (D) MHV-infected CCL9.1 cells subjected to 62.5 μg/mL of quercetin. Absence of obvious CPE in (C) indicates neutralization of MHV infectivity.

Figure 3. Neutralization of MHV infectivity by cinanserin hydrochloride on 2 dpi.
(A) Uninfected control of CCL9.1 cells. (B) MHV-infected CCL9.1 cells control. (C) MHV-infected CCL9.1 cells treated with 31.25 μg/mL of cinanserin. (D) MHV-infected CCL9.1 cells treated with 62.5 μg/mL of cinanserin. (E) MHV-infected CCL9.1 cells treated with 125 μg/mL of cinanserin. Absence of obvious CPE in (D) and (E) indicates neutralization of MHV infectivity.
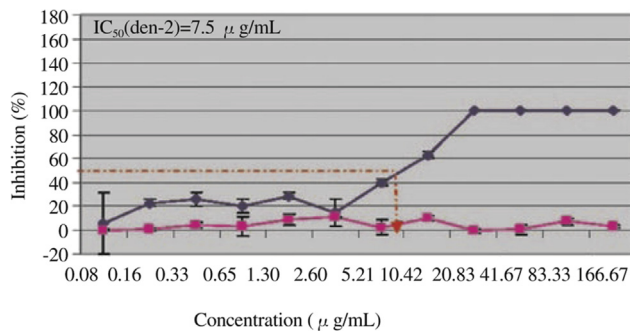
Figure 4. The EA fraction of *H. cordata* inhibits DENV-2 infectivity but is not cytotoxic to BHK-21 cells.

The EA fraction of *H. cordata* was tested at concentrations of 166.67 µg/mL down to 0.08 µg/mL. The upper plot reveals a distinct trend of DENV-2 inhibition, with the IC_{50} being 7.5 µg/mL, and complete viral inhibition being achieved at concentrations of 20.83 µg/mL and above. The lower plot shows that no cytotoxicity to BHK-21 cells was evident at various concentrations of the EA fraction tested.

Table 2

IC_{50}, CC_{50} and selectivity indices of *H. cordata* fraction, flavonoids, and their combination against DENV-2.

Sample	IC_{50} (µg/mL)	CC_{50} (µg/mL)	Selectivity index
EA fraction	7.50	NC[a]	>22.22[b]
Quercetin	176.76	155.38	0.88
Quercitrin	467.27	NC[a]	>1.07[c]
Quercetin + Quercitrin	158.21	270.00	1.71
Rutin	NIL	NC[a]	NIL

[a] NC: No cytotoxicity at all concentrations tested against BHK-21 cells.
[b] Highest selectivity index of EA fraction (*i.e.*, lowest concentration for viral inhibition coupled with absence of cytotoxicity). [c] Selectivity index with absence of cytotoxicity.

of *H. cordata* extract showed no mortality and no significant weight loss (data not shown). Moreover, all mice appeared to be active and well-groomed before euthanasia. From the histopathological sections of all major organs stained with H&E, there was no significant difference between the control group and all the test groups, as exemplified in Figure 5 which depicts the control group compared with the group fed with the highest dose of 2 000 mg/kg.
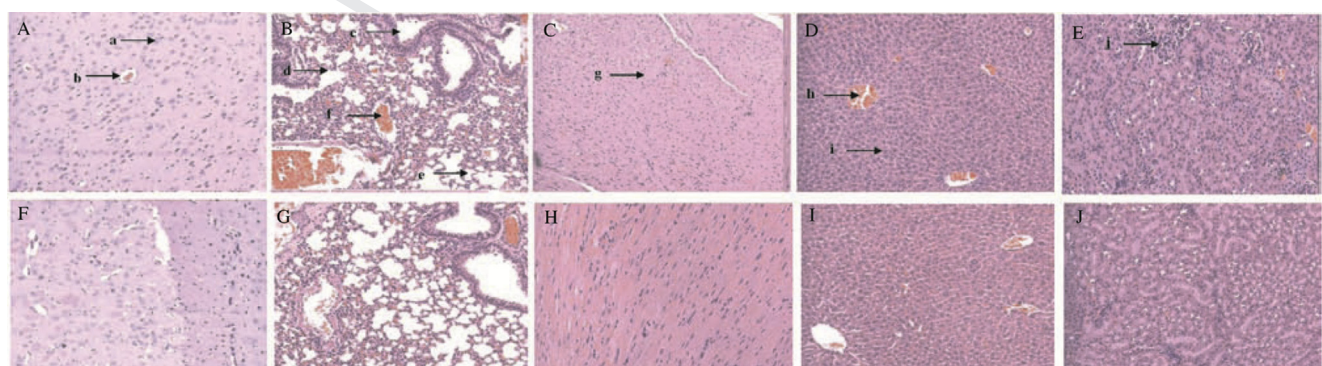
4. Discussion

The experimental strategy in this study was to investigate the prophylactic antiviral effects since the compounds were allowed to interact with the viruses for 1 h before introduction into the cells. This approach was employed as we previously found no therapeutic effect when the compounds were added after prior infection of cells with DENV [13].

Overall, this study demonstrated that EA fraction of *H. cordata* and its flavonoid component, quercetin, could inhibit both MHV and DENV-2 *in vitro*. This was followed by quercitrin which could inhibit DENV-2 but not MHV, whereas rutin did not exert any inhibitory effect on both viruses. We and others have provided evidence to show that different flavonoid components in the EA fraction of *H. cordata* exert varying degrees of antiviral activity against different viruses. Our findings corroborate previous evidence that among the flavonoid components, quercetin is the most effective against DENV-2 [13]. Wleklik *et al.* emphasized that there is a structural basis for the distinct differences in antiviral activities of flavonoids [21]. Hence, it is noteworthy that being the most bioactive, quercetin possesses the hydroxyl group at the R2 position compared to the other two flavonoids tested, *i.e.*, rhamnose in quercitrin and rubinose in rutin [22].

Quercetin is an aglycone present at high concentration in onions. This compound has virucidal activity against enveloped viruses such as mengovirus, herpes simplex, parainfluenza type 3, pseudorabies, respiratory syncytial, and Sindbis viruses [23–26]. Quercetin is able to inhibit H^+-ATPase of lysosomal membrane and thus prevent virus coat removal [15]. Moreover, quercetin exhibits significant inhibitory effects on the ATPase of multidrug resistance-associated proteins, thus increasing the bioavailability of anticancer and antiviral drugs *in vivo* [27]. Hence, quercetin can be considered for its potential efficacy in antiviral drug therapies.

Quercitrin (quercetin-3-L-rhamnoside) and rutin (quercetin-3-rutinoside) occur as glycosides. Quercitrin appears to show the highest content in fingerprint analysis of *H. cordata* by HPLC [14]. Among the flavonoids, quercetin also exerts the highest activity on HSV-1, whilst rutin has no effect at all [21]. This characteristic implies that the substitution or addition of free hydroxyl group at certain positions may culminate in a decreased or even completely abolished antiviral effect. Such

Figure 5. The EA fraction of *H. cordata* does not cause acute oral toxicity in mice.

Representative histopathological sections of major organs stained with H&E for the vehicle control group (images A–E) and test group fed with 2 000 mg/kg of EA fraction of *H. cordata* (images F–J). (A, F) Brain with (a) nuclei of neurons; (b) blood capillary. (B, G) Lung with (c) respiratory bronchiole; (d) alveolar duct; (e) alveoli; (f) blood vessel. (C, H) Heart with (g) cardiac muscle. (D, I) Liver with (h) central vein; (i) hepatocytes. (E, J) Kidney with (j) glomeruli.

structural-activity relationships can lead to the design and development of more active and less toxic flavonoids with appropriate pharmacokinetic properties. Hence, they may pave the way for novel compounds that can match or even exceed the efficacies of existing antiviral drugs [26,28,29].

Another interesting aspect was the enhancement of anti-DENV-2 activity of quercetin when combined with quercitrin. Besides augmenting the antiviral effect, this combination also reduced the cytotoxicity relative to that induced by quercetin alone. This characteristic reiterates that combinations of components with greater anti-dengue efficacy but lower toxicity are potentially promising, as discovered previously using combinations of quercetin, chlorogenic acid and/or hyperoside [13]. Indeed, several reports have already documented the synergistic antiviral effects of combinations of individual flavonoids against viral pathogens, such as herpesviruses and fowlpox virus [30,31].

Although cinanserin was able to inhibit MHV at 31.25 μg/mL on 1 dpi and 62.5 μg/mL on 2 dpi, its effect was relatively short-lived and it could not inhibit MHV from 3 dpi onwards. In comparison, however, we observed that the EA fraction and quercetin yielded a longer-lasting antiviral effect of up to 6 d. This finding may be explained by superimposition of the two 3C-like proteinases of MHV and SARS CoV which illustrates that certain amino acid residues residing within 7 Å of the active site (C145) are different (not shown). Thus, these amino acid disparities may influence recognition and binding of cinanserin hydrochloride to the active site and may also lead to its lower affinity for 3C-like proteinase of MHV than SARS-coronavirus. Competitive binding of cinanserin hydrochloride to the active site of 3C-like proteinase hinders the processing of the precursor polyprotein to generate functional replicase necessary for viral replication [32–34].

Importantly, the EA fraction of *H. cordata* was capable of inhibiting both DENV-2 and MHV *in vitro* at relatively low concentrations with negligible cytotoxicity and very good selectivity indices. Moreover, the EA fraction was also more potent than all the individual compounds tested (including their combination) and did not cause any cytotoxicity to mammalian kidney (BHK) and liver (CCL9.1) cell lines *in vitro*, nor any acute toxicity or pathology of major murine organs *in vivo*. This feature reiterates the synergistic effects of the combination of multiple flavonoids and other constituents within *H. cordata* that confer optimal antiviral activities and minimal toxicity [35], in keeping with the majority of successful traditional Chinese medicine.

Our study lends support to the accumulating literature that the EA fraction of *H. cordata* indeed represents a highly promising prophylactic agent against coronaviruses and dengue viruses [36–38]. To complement this study, future *in vivo* studies are warranted to assess the efficacy of the EA fraction of *H. cordata* against coronaviruses and dengue viruses using the relevant mouse models [39]. However, further *in vivo* experiments are necessary to further assess the long-term safety of *H. cordata* as an antiviral agent in biological systems, including acquiring crucial information on its adsorption, distribution, metabolism and elimination. To address the important issue of batch-to-batch variation, greater efforts should also focus on the quality control of extracts derived from this medicinal plant. Finally, the mechanisms of action of the EA fraction and its constituent flavonoids against viruses at the molecular level need to be further elucidated [40].

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